Identification of MMP-1 as a putative breast cancer predictive marker by global gene expression analysis

Indira Poola[1], Robert L DeWitty[2], Josephine J Marshalleck[3], Rakesh Bhatnagar[4], Jessy Abraham[1] & LaSalle D Leffall[2]

Breast cancer is the second leading cause of cancer death for women in the United States. In 2005, about 215,000 cases of invasive breast cancer (IBC) and 50,000 cases of ductal carcinoma *in situ* will be diagnosed and 40,000 women will die of IBC in the US[1]. Yet there is presently no molecular marker that can be used to detect a precancerous state or identify which premalignant lesions will develop into invasive breast cancer. Here we report the gene expression analysis of atypical ductal hyperplastic tissues from patients with and without a history of breast cancer. We identify MMP-1 as a candidate marker that may be useful for identification of breast lesions that can develop into cancer.

To identify putative markers of IBC, we compared the global gene expression profiles of six ADHC tissues (from patients with atypical ductal hyperplasia (ADH) who had cancer concurrently, who had cancer before diagnosis of ADH or who developed cancer subsequently) against ten ADH control tissues from patients who had no cancer before ADH diagnosis and did not develop cancer in the 5 years after diagnosis. ADH samples were selected for this analysis because women with ADH have approximately 5 times higher incidence of IBC than women who did not have ADH[2,3]. All ADH samples were derived from biopsy tissues and ADHC samples were derived from either biopsy or mastectomy tissues (for details see **Supplementary Table 1** online). The fresh tissues for expression analyses were obtained by harvesting the lesions immediately adjacent to the tissue used for histological diagnosis and were considered to be representative of the tissue utilized for diagnosis. We performed four analyses for each group on U133A (Affymetrix) gene chip using individual or pooled RNA samples (GEO accession number GSE2429).

By comparing the global gene expression profiles of ADHC and ADH, we identified about 540 differentially expressed genes in ADHC and statistically analyzed this expression (**Supplementary Methods** online). The raw data, comparisons and differentially expressed genes are presented in **Supplementary Table 2** online. Validation of the expression of selected gene mRNAs by real-time RT-PCR, hierarchical clustering and over-represented gene ontologies are shown in **Supplementary Fig. 1**, **Supplementary Fig. 2** and **Supplementary Table**

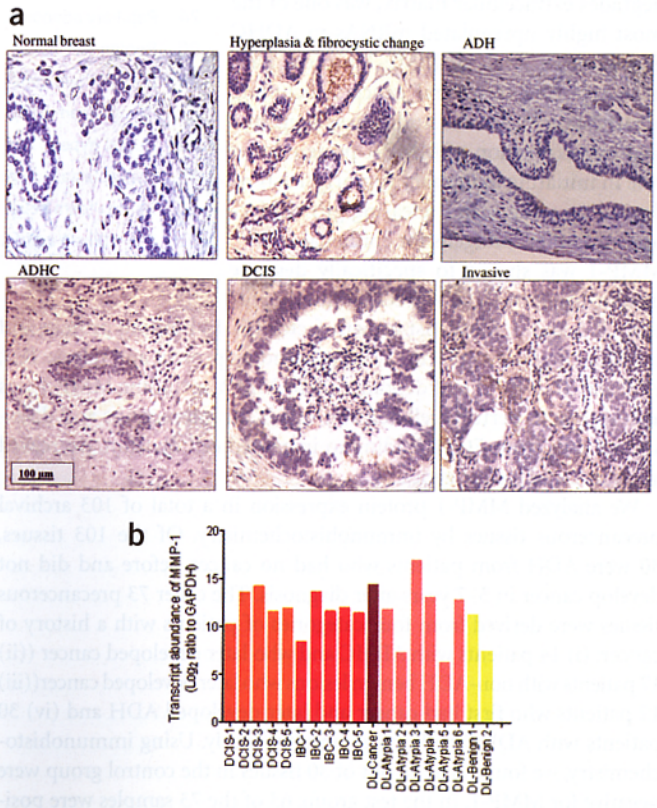

Figure 1 MMP-1 protein levels in breast tissues by IHC, *MMP1* mRNA in ductal lavage by quantitative real-time RT-PCR. (**a**) Paraffin-embedded tissue sections were immunostained by investigators blinded to the experimental conditions using standard procedures with monoclonal antibodies against MMP-1. Representative tissues stained from normal, MMP-1–positive non-ADH benign tissue (hyperplasia with fibrocystic change), ADH, ADHC, DCIS and IBC are shown (magnification, ×100). MMP-1 staining could be seen both in ductal epithelial cells and stroma. (**b**) Feasibility of detecting *MMP1* mRNA was tested in a total of 31 ductal lavage samples by quantitative real-time RT-PCR using Assays-on-Demand reagents from Applied Biosystems, Inc. The ductal lavage samples were obtained from donors using InDuct breast micro-catheters from Cytyc and total RNAs were isolated using Qiagen RNeasy Micro Kits. *MMP1* transcript levels in nine positive ductal lavage, five representative DCIS and Stage I IBC samples are shown as histograms. Six samples diagnosed as having atypia (DL-Atypia 1–6) and two samples diagnosed as benign (DL-Benign 1–2) showed comparable transcript levels as DCIS and Stage I samples. Ductal lavage collection procedure was approved by the Howard University Institutional Review Board Committee.

[1]Department of Biochemistry and Molecular Biology, Howard University College of Medicine, Washington, D.C. 20059, USA. [2]Surgical Oncology and [3]Pathology, Howard University Hospital, Washington, D.C 20060, USA. [4]Biology Department, University of Alberta, Edmonton, Canada, T6G2E9. Correspondence should be addressed to I.P. (ipoola@howard.edu).

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3 online, respectively. Among the 540 genes identified, several were previously reported to be differentially expressed in breast cancer[4] and include 10 (*ANKT*, *CENPA*, *TOPK*, *RRM2*, *TOP2A*, *NEK2*, *CDKN3*, *BUB1*, *BIRC5* and *CKS2*) of the 38 genes that were previously reported to be upregulated during breast cancer progression[5].

The differentially expressed genes in ADHC encode for proteins that regulate at least 11 major categories of cellular functions (**Supplementary Table 4** online). The most significantly ($P < 10^{-4}$) upregulated mRNAs were those encoding proteins involved in: (i) regulating cell cycle check points((ii) increasing nucleic acid levels((iii) increasing estrogen levels((iv) degrading extracellular matrix (ECM)((v) maintaining cell polarity and architecture((vi) increasing cellular proliferation((vii) inhibiting apoptosis and (viii) EGFR signaling. Other elevated mRNAs include those encoding for proto-oncogenes and those expressed by B and T lymphocytes. The most significantly ($P < 10^{-4}$) downregulated mRNAs were those encoding tumor suppressors and growth factor binding proteins.

MMP1, which encodes an enzyme that degrades extracellular matrix, was one of the most highly upregulated mRNAs in ADHC and elevated levels of MMP-1 protein have been reported in several cancers including breast cancer[6,7]. Animal studies have suggested that overexpression of MMP-1 protein has a role in initiating mammary tumorigenesis by degrading stroma and releasing growth factors and other mitogens for epithelial cells[8,9]. MMP-1 was shown to specifically degrade insulin-like growth factor (IGF) binding proteins 2, 3 and 5, fibroblast growth factor (FGF) binding protein and transforming growth factor (TGF)-β binding protein and release IGF, FGF and TGF-β[10]. ECM degradation by MMPs including MMP-1 was also shown to perturb cell-cell and cell-ECM interactions and disassociate cells from ECM, leading to increased cell division, decreased apoptosis and tumorigenesis[8].

Table 1 ***MMP1*** **mRNA in ductal lavage samples by quantitative real-time RT-PCR**

No.	Cytological diagnosis	Nipple discharge	MMP-1 presence
1.	Benign with multinucleated histiocytic giant cells and rare mixed inflammatory cells	No	Positive
2.	Benign, mixed inflammatory cells	Yes	Negative
3.	Cancer	Yes	Positive
4.	Benign, fragments of hyperplasic duct-lining cells	Yes	Negative
5.	Benign	No	Negative
6.	Fibrocystic disease with apocrine metaplasia	Yes	Negative
7.	Benign, honeycomb appearing nests of hyperplastic ductal cells	Yes	Negative
8.	Benign	Yes	Negative
9.	Benign	Yes	Negative
10.	Benign, foam cells and mixed inflammatory cells	Yes	Negative
11.	Atypical epithelial cells	Yes	Positive
12.	Hyperplasia to mild atypia	Yes	Negative
13.	Benign	No	Negative
14.	Benign, foam cells and inflammatory cells	Yes	Negative
15.	Benign, foam cells and mixed inflammatory cells	Yes	Negative
16.	Hyperplasia with atypia	Yes	Positive
17.	Benign, foam cells and mixed inflammatory cells	Yes	Negative
18.	Hyperplasia with mild atypia, foam cells & mixed inflammatory cells	Yes	Positive
19.	Hyperplasia, foam cells and mixed inflammatory cells	Yes	Negative
20.	Benign, foam cells and mixed inflammatory cells	Yes	Positive
21.	Hyperplasia to atypia, foam ductal/histiocytic cells	Yes	Positive
22.	Hyperplasia to atypia	Yes	Positive
23.	Benign, foam cells	No	Negative
24.	Papillary adenoma, hyperplasia, atypia and foam cells	Yes	Positive
25.	Benign foam cells	Yes	Negative
26.	Benign foam cells	No	Negative
27.	Benign foam cells	No	Negative
28.	Ductal hyperplasia	No	Negative
29.	Hyperplasia with mild atypia	No	Negative
30.	Benign foam cells	Yes	Negative
31.	Hyperplasia and foam cells	Yes	Negative

We analyzed MMP-1 protein expression in a total of 103 archival precancerous tissues by immunohistochemistry. Of the 103 tissues, 30 were ADH from patients who had no cancer before and did not develop cancer in 5–7 years after diagnosis. The other 73 precancerous tissues were derived from four categories of patients with a history of cancer: (i) 14 patients with ADHC and who later developed cancer ((ii) 17 patients with non-ADH benign lesions who later developed cancer((iii) 12 patients who first had cancer and later developed ADH and (iv) 30 patients with ADHC and cancer simultaneously. Using immunohistochemistry, we found that 26 out of 30 tissues in the control group were negative for MMP-1. In the test group, 63 of the 73 samples were positive. Details about tissues used and scoring of MMP-1 staining intensities, which were graded in comparison to an arbitrary score of 5 in both epithelial cells and stroma of invasive cancer tissues, are presented in **Supplementary Table 5** online. Statistical analysis of the above data showed a significant ($P = 2.7 \times 10^{-9}$) association of MMP-1 protein expression with precancerous tissues obtained from patients with a history of cancer by both chi-squared and Fisher exact tests. Representative micrographs of normal, MMP-1–positive ductal hyperplasia with fibrocystic changes, ADH, ADHC, IBC and ductal carcinoma *in situ* (DCIS) tissues are presented in **Fig. 1a**. Both stroma and epithelial cells were positive for MMP-1. More intense staining was observed in stroma in all the tissues. Among various samples tested, most intense staining was observed in ADHC from patients who simultaneously had cancer and weakest was in non-ADH benign lesions (**Supplementary Table 5** online).

We next tested the feasibility of detecting *MMP1* mRNA in a total of 31 ductal lavage samples by real-time RT-PCR. Of the 8 samples diagnosed as atypia, 6 were positive and 20 of the 22 diagnosed as benign were negative for *MMP1* mRNA (**Table 1**). *MMP1* mRNA levels in ductal lavage samples were comparable to the levels detected in IBC and DCIS samples (**Fig. 1b**). These results show that *MMP1* mRNA can be detected in cells obtained from ductal lavage.

The data presented here suggest that MMP-1 could potentially be used as a diagnostic marker for screening ADH and non-ADH benign tissues and identifying patients with lesions that may develop into cancer. It also may prove useful for screening women with no lesions using samples of ductal cells obtained by procedures such as ductal lavage collection and random periareolar fine needle aspiration and identifying

those at risk of cancer development. Finally, MMP-1 upregulation in a precancerous lesion may suggest a need for treatment similar to IBC.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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